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Pathogenicity and Antibiotic Sensitivity of Pathogenic Flora Associated with the Gut of Blue Swimming Crab, *Portunus pelagicus* (Linnaeus, 1857)

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Abstract: *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Pseudoalteromonas piscicida*, *Staphylococcus epidermidis* and *Micrococcus luteus* were isolated from the gut of blue swimming crab, *Portunus pelagicus* captured from Strait of Tebrau Johor Malaysia and studied for pathogenicity against the Zoea-1 (Z1 stage) of *P. pelagicus*. Pathogenic isolates *V. harveyi* and *P. piscicida* resulted in 100% mortality at 10^6 cfu mL^{-1} and 10^5 cfu mL^{-1} after 24 h and 72 h post dose. Conversely, *V. parahaemolyticus* produced 100% deaths at inoculation 10^6 cfu mL^{-1} after 72 h post dose. Cumulative mortality was observed rising with the increase in dose potency of pathogens. *S. epidermidis* and *M. luteus* detected with feeble pathogenic characteristics. The LD_{50} of *V. harveyi* was 1.2×10^3 cfu ML^{-1} (24 h), *V. parahaemolyticus* was 9.6×10^5 cfu mL^{-1} (72 h), *P. piscicida* was 9.8×10^3 cfu mL^{-1} (24 h) and *S. epidermidis* was 9.8×10^5 cfu mL^{-1} (72 h). The mean differences among various pathogenic doses were statistically significant ($p<0.05$). Susceptibility tests of total 662 isolates were under taken including *V. harveyi* (n = 180), *V. parahaemolyticus* (n = 180) and *P. piscicida* (n = 119), isolates showed mixed trend as multiple resistance and sensitive to antimicrobial agents tested while *S. epidermidis* (n = 88) and *M. Luteus* (n = 95) were sensitive to all antibiotics tested. *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* did not show 100% resistance to any of the antibiotics tested. From the results of 14 antibiotics tested, we observed that the highest frequency of single drug resistance in *V. harveyi* was Streptomycin (89.44%) and sensitive to chloramphenicol (70.55%). Similarly, the highest frequency of single-drug resistance in *V. parahaemolyticus* was to kanamycin (92.78%) and sensitive to chloramphenicol (93.33%) and *P. piscicida* was to penicillin (80.67+19.33% intermediate but no sensitive) and sensitive to gentamicin (98.32%). Infections caused by antibiotic resistant pathogens have serious consequences and therapeutic use of tested antibiotic is questionable in larviculture of *P. pelagicus*.

Key words: *Portunus pelagicus*, pathogenicity, susceptibility, antimicrobial, therapeutic, larviculture

INTRODUCTION

One of the major problems in aquaculture is disease (Austin and Allen-Austin, 1985). Some researchers consider that bacterial diseases are a major cause of mortalities in hatcheries (Gomez-Gil *et al*., 2000). Bacterial diseases are mainly caused by opportunistic (facultative) bacterial pathogens which can reside in the environment or on/in apparently normal fish (latent carriers) (Wedemeyer, 1996). Pathogenic *Vibrio* species are a major cause of disease problems in aquaculture (Austin and Austin, 2007; Cano-Gomez *et al*., 2009; Ruangpan and Kitao, 1991; Vandenberghe *et al*., 2003). *V. harveyi* has been widely recognized as a principal pathogen of many commercially cultured invertebrate species the world over. In the black tiger shrimp, *Penaeus monodon* for example, the mortality of larval stages (protozoea to postlarvae) in the hatcheries often reaches 100% (Lavilla-Pitogo *et al*., 1990). Outbreaks caused by *V. harveyi* have been reported in many marine fishes (Sunaryanto and Mariam, 1986; Soffientino *et al*., 1999; Zhang and Austin, 2000; Won *et al*., 2006). *V. parahaemolyticus* has emerged as an important fish pathogen and there are many reports on the involvement of this bacterium in shrimp vibriosis (Ruangpan and Kitao, 1991; Xu *et al*., 1994; Chanratchakool *et al*., 1995; Alapide-Tendencia and Dureza, 1997). *V. parahaemolyticus* caused massive epidemics among shrimps in Thailand (Nash *et al*., 1992)

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and the Philippines (Lavilla-Pitogo *et al*., 1990). The pathogenic character of *P. piscicida* bacterium is poorly documented in literature. An isolate *P. piscicida* Cura-d was associated with the highest mortality of both *Amphiprion clarkii* (Bennett) and *Amphiprion curacao* (Bloch) eggs. The majority of the eggs die within 24-36 h (Nelson and Ghiorse, 1999). Hansen *et al*. (1965) reported that *P. piscicida* appeared to be toxic to certain species of fish including the fiddler crabs, *Uca pugnas* and *Uca pugilator*. A toxic syndrome appears within a few hours and death follows quickly after neuromuscular effects appear. *S. epidermidis* and *M. luteus* have also been reported as fish pathogens but to date literatures does not offers any information for the pathogenicity mechanism of these bacteria in blue swimming crab *Portunus pelagicus* larvae. Microbial infections have been a major concern of aquaculture worldwide and gut flora of fish have been researched by many researchers but to date, no information on pathogenic microbes with the gut of *P. pelagicus* and their pathogenic role with the larval survival and their response to antibiotic susceptibility has been documented. Uaboi-Egbenni *et al*. (2010) examined pathogen in the gut of swimming crab, *Callinectes* sp. for the purpose of public health and epidemiological implications not for aquaculture.

In the present study, we research on the pathogenicity and antimicrobial susceptibility of microbes isolated from the gut of *P. pelagicus*. This study was conducted in purview of aquaculture aspect to evaluate the possible pathogenic role of isolated pathogens with larval survival and effectiveness of common antibiotic used for aquaculture.

MATERIALS AND METHODS

Sample collection and sampling site: The present study was conducted in the marine hatchery and the laboratory of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia. Every month 20-30 crab samples were collected from Strait of Tebrau, Johor, Malaysia, (1°22'N and 103°38'E) with different Body Weight (BW) and Carapace Width (CW) (for 12 months from December 2009 to November 2010. The collected samples were transferred into 40 L round polythene tanks equipped with aeration. Salinity at site was measured between 31-33 ppt. To avoid contamination, the samples kept in same water until they dissected for microbes study.

Experimental design: A two-factor experimental design was used to evaluate; the pathogenic effect of the gut pathogens on larvae of *P. pelagicus* through challenge doses and to determine the antibiotic susceptibility of pathogens associated with the gut.

Larvae rearing: Larvae of *P. pelagicus* were produced in marine hatchery of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia for pathogenic challenge tests.

Bacteriological study

Segregation of the gut: Prior to segregation of the gut, crabs specimen were randomly collected from the sample stock and bathed in 10% formalin for 20 min. Subsequently they were again washed with fresh tap water for 5 min and finally washed with sterile de-mineralized water in order to get rid of surface microflora. Sterile dissecting materials were used for this purpose. Aseptically crabs specimen were dissected, the whole gut was removed and pulverized with pestle and mortar vigilantly and mixed with sterile Sea Water (SW) to prepare inoculum. Bacteria isolation were carried out by serial dilution (up to 3 fold). For the present study every month 15 female crab specimens and a total 180 female crab specimens (for 12 months) studied for microbes associated with the gut.

Bacterial culture: Different enrichment and selective culture media were used to determine the accumulation of pathogenic bacteria in the gut of the *P. pelagicus*. The medium employed were among others, Thiosulphate Citrate Bile Salts (TCBS. Difco, USA), MacConkey agar (Merck Germany), Nutrient agar (Merck Germany), Marine agar (Merck Germany) and GSP agar (Biolab Hungary). All media were prepared in sterilized seawater (31±2 ppt). All plates were incubated at 37°C for 24 h. Pure cultures were obtained by repeated streaking method.

Identification and characterization micro flora

Total genomic DNA extraction: Total genomic DNA of all compost samples was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instruction. Briefly, 1 mL of overnight culture was centrifuged for 2 min at 13, 000 rpm. The cells then suspended in 480 μL of 50 mM EDTA and 120 μL of lytic enzyme before incubation at 37°C for 60 min. The mixtures were then centrifuged for 2 min at 13, 000 rpm and the supernatant was removed. Nuclei Lysis Solution 600 μL was added to the pellet and mixed gently by pipetting. The mixture was then incubated for 5 min at 80°C and left over to cool down at room temperature. RNase Solution 3 μL was added to the mixture and

incubated at 37°C for 15-60 min, subsequently cooled at room temperature. Then 200 μL of protein precipitation solution was added to mixture, vortexed and incubated on ice for 5 min followed by centrifugation at 13, 000 rpm for 3 min and supernatant was transferred to a clean tube containing 600 μL of room temperature isopropanol and mixed properly. The mixture was centrifuged for 2 min at 13, 000 rpm and the supernatant decanted and then 600 μL of 70% ethanol (room temperature) was added, mixed and centrifuged for 2 min at 13, 000 rpm. The ethanol then aspirated and the pellet air-dried for 10-15 min. After that DNA pellet rehydrated in 100 μL of rehydration solution for 1 h at 65°C or overnight at 4°C.

Polymerase Chain Reaction (PCR) amplification of 16S rDNA: The 16S ribosomal DNA was amplified by PCR using bacterial universal primers 27F (5'-AGAGTTTGAT CCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTG TTACGACTT-3'). The PCR reaction was performed in a Bio Thermal cycler (Bio-Rad, USA) with an initial denaturing step at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min and ended with a final extension step of 72°C for 15 min. The PCR products were electrophoreses in 1% agarose gel stained with 1 μg mL^{-1} of ethidium bromide and was visualized using Alpha Imager gel documentation system (Alpha Innotech, UK). Sequences obtained were analysed and compared with sequences from GenBank using BLASTn NCBI citation (http://blast.ncbi.nlm.nih.gov).

Pathogenicity test of gut isolates: About 1 day hatch larvae of Zoea-1 stage (Z1) of *P. pelagicus* were used to evaluate the pathogenicity of the gut isolates with slight modification according to Villamil *et al.* (2003). Larvae were fed on a mixture of live prey composed of 30-40 rotifers mL^{-1} (*Brachionus* sp.) with *Nanochloropsis* sp. (8×10^5 cells mL^{-1}). Challenge experiments were conducted in 1 L transparent aquaria containing Sterilised Seawater (SW) equipped with aeration. Prior to exposing to challenges, energetic larvae were acclimated in 2 L sterilized sea water with similar parameters as in hatching tanks and challenge aquaria. Water from acclimation aquaria was sucked out with a small pipe fixed with 10 μ net at the suction end and new water was poured in with other pipe. This practice was exercised in order to minimise the bacterial load with larvae adhering from hatching tank water. All larvae were washed in this way using 5 L sterilised seawater. Leading pathogenic bacteria isolated from the gut of *P. pelagicus* were cultured in Marine Broth (prepared with seawater) at 37°C for 24 h under agitation (80 rpm). Bacterial cells were subsequently collected by centrifugation (7500 g, 5 min) rinsed with sterile seawater and re-suspended in sterile seawater. Bacterial suspension was measured to $OD_{630\ nm}$ relationship previously established. In the experiments, isolates were added to experimental aquaria at a final 10 fold concentration inoculation of 10^2, 10^3, 10^4, 10^5 and 10^6 cfu mL^{-1}. In all experiments, no-pathogen controls were employed. All experimental challenges were carried out in triplicate and an initial number of 20 larvae L^{-1} were placed in each 1 L aquaria and mortality (%) was determined by direct counting of larvae after 24, 48 and 72 h of post challenge, slight modification to Ricque-Marie *et al.* (1998) (Total number of larvae died/Initial number of larvae stocked×100). Larvae were divided into 5 groups and one control. Each group was tested with one isolate and total five isolates were tested. Three aquaria for challenge dose and three controls hence total six aquaria for each concentration and for five isolates for single experiment therefore, 30 aquaria were used and for all experimental challenges and total 150 aquaria were tested. Water temperature, dissolved oxygen, pH and salinity were monitored accordingly using YSI 556 MPS (USA) multi parameter equipment. LD_{50} was calculated by the method described by Miller and Tainter (1944).

Antibiotic sensitivity test: Susceptibility of the 662 isolates including *V. harveyi* and *V. parahaemolyticus* (n = 180, respectively), *P. piscicida* (n = 119), *M. luteus* (n = 95) and *S. epidermidis* (n = 88) to various antibiotics was determined on Mueller-Hinton Agar (MHA) (Merck) by the disc diffusion method described by Bauer *et al.* (1996). Leading pathogenic bacteria isolated from the gut of *P. pelagicus* were cultured in Marine Broth prepared in seawater incubated for OD growth at 37°C for 24 h under agitation (80 rpm). Bacterial suspension was measured at $OD_{630\ nm}$ relationship previously established.

Overnight young cultures of bacteria from Marine broth were inoculated on to the surface of MHA using sterile cotton swabs. The inoculum was allowed to dry for 5 min. Antibiotic impregnated discs (4 or 5 discs for each plate) were then placed aseptically on to the inoculated agar plates away from the edge at equal distance and sufficiently separated from each other to avoid overlapping of the zone of inhibition. The antibiotic discs used were: Co-trimoxazole (trimethoprim and sulfamethoxazole) (25 μg), Erythromycin (15 μg), streptomycin (10 μg), oxytetracycline (30 μg), ampicillin (10 μg), nystatin (mycostatin) 100 units, cholramphenicol (30 mg), gentamicin (100 μg), penicillin (10 μg), kanamycin (30 μg), furazolidone (50 μg), ciprofloxacin (10 μg),

rifampicin (5 μg) and neomycin (30 μg). The plates were sealed and incubated at 37°C overnight. Then, the inhibition zone was measured and the bacteria were classified into either susceptible or resistant or intermediate as referred to an interpretative table of Sensi-Disc Antimicrobial Susceptibility Test Discs; Approved Standard, 1996.

Statistical analysis of data: Means of different concentrations and morality were compared by ANOVA analysis of variance using statistical software (SPSS 16.0 for windows). Post hoc test was carried out using Duncan multiple range tests if they were significant.

RESULTS

Under the present study a total 180 female crabs were studied for pathogenic microbes. *V. harveyi* and *V. parahaemolyticus* were found as dominant pathogen with all female specimens. Whereas *P. piscicida* in 119 female crab guts followed by *M. Luteus* in 95 females and *S. epidermidis* in 88 female guts. The isolates were identified using *16S rRNA* gene sequences analyses. PCR amplification of *16S rRNA* gene sequence result divulged the diversity of pathogenic floral consortium in the gut; it includes *V. harveyi*, *V. parahaemolyticus*, *P. piscicida*, *S. epidermidis* and *M. luteus*. Sequences obtained were analysed and compared with sequences from data base GenBank using BLASTn (megablasn) National Center for Biotechnology Information (NCBI) USA online data bank citation (http://blast.ncbi.nlm.nih.gov). Results obtained from one female gut isolates was set as precedent and shown in Table 1. The sequence results from GenBank showed similarity with various strains of same species among all isolates (Table 2). Morphological, biochemical and physiological tests of all isolates were carried out earlier (Talpur *et al*., 2011).

Besides *16S rRNA* gene sequences analyses, Burgey's manual of determinative bacteriology, morphological, physiological and biochemical characteristics of bacteria, BD BBL Crystal Identification systems (Becton, Dickinson and company, USA), API Staph kit systems were also used for identification of bacteria during the present study.

Larvae (Z1 stage) were exposed to 10 fold concentration ranging from 10^2-10^6 cfu mL^{-1} and larval mortality was observed for 72 h and LD_{50} was calculated. The results of cumulative mortalities of gut isolates showed the *V. harveyi*, *P. piscicida* and *V. parahaemolyticus* were more pathogenic while *S. epidermidis* and *M. luteus* were observed as weak pathogens. *V. harvey*i was more pathogenic at dose 10^6 cfu mL^{-1} and killed all (100%) larvae within 24 h post dose and 10^5 cfu mL^{-1} resulted in mortality 91.67, 98.33% after 24 and 48 h post dose, respectively. Experimental doses of *V. harveyi* at inoculation10^2, 10^3, 10^4 and 10^5 cfu mL^{-1} produced mortality 53.33, 61.67, 93.33 and 100%, respectively after 72 h post dose. Moreover, doses 10^6 and 10^5 cfu mL^{-1} were observed highly pathogenic and resulted 100% mortality after 24 and 72 h, respectively. This organism showed pathogenicity in all experimental challenges and with the increasing dose concentration, it reflected more deaths at all intervals after post dose (Table 3). *V. parahaemolyticus* at inoculation dose 10^2, 10^3, 10^4 and 10^5 cfu mL^{-1} produced mortality 41.33, 48.33, 56.67 and 78.33%, respectively. Higher

Table 1: Identification based on16S rRNA gene sequencing result of isolates from the gut of *P. pelagicus*

Species	GenBank accession No	Strain	Max. identity (%)
V. harveyi	GU262992.1	090212	99
V. parahaemolyticus	EU660326.1	CM12	99
P. piscicida	AB090233.1	O-7	99
M. luteus	FJ816022.1	G3-6-08	99
S. epidermidis	FJ605382.1	AceN-1	99

Table 2: The 16S rRNA gene sequencing result show similarity among isolates from the gut of *P. pelagicus*

Species	Similarity at 99% identity to strains of same species									
V. harveyi	S090801	LA08005	LA08008	XC08001	20060928-1	S35 16S	S20	VS1 16S	LMG4404T	NCIMB1280T
V. parahaemolyticus	RIMD 2210633	UST040801-006	6	CT12	CT11	V4	2210633	CM11	R22	MM21
P. piscicida	IAM 12932	NCIMB645	S1948	S1845	S1607	S2047	S1947	L2	1314	S1925
M. luteus	NSM12	CV39	HM1	PCSB6	ZFJ-12	EHFS1_S01Hd	Ballarat	EHFS1_S18Hb	AUH1	BQN1T-03d
S. epidermidis	MB	BBAR7-13d	BQN3N-03d	BQER2-01	BBN3N-03d	BBN1Ld-02a	NM62-4	G58-1	IMAU:80829	BBN1B-

Table 3: Cumulative mortality (%) in *P. pelagicus* larvae (Zoea1 Z1) after exposure to different doses (cfu mL^{-1}) of *V. harveyi* isolate from the gut (Mean±SD)

Dose	Mortality (%)					
	Control	24 h	Control	48 h	Control	72 h
10^2	16.67±2.89	41.67±5.77	18.33±2.89	46.67±5.77	23.33±2.89	53.33±2.87
10^3	13.33±2.89	48.33±2.89	16.66±2.89	55.00±5.00	21.67±2.89	61.67±2.89
10^4	15.00±5.00	66.67±2.89	21.67±2.89	78.33±2.89	23.33±2.89	93.33±2.89
10^5	15.00±5.00	91.67±2.89	20.00±5.00	98.33±2.89	25.00±5.00	100.00±0.00
10^6	15.00±5.00	100.00±0.00	16.66±2.89	100.00±0.00	18.33±2.89	100.00±0.00

SD = Standard Deviation

Table 4: Cumulative mortality (%) in *P. pelagicus* larvae (Zoea1 Z1) after exposure to different doses (cfu mL^{-1}) of *V. parahaemolyticus* isolate from the gut (Mean±SD)

	Mortality (%)					
Dose	Control	24 h	Control	48 h	Control	72 h
10^2	11.67± 2.89	26.67±2.89	18.33±2.89	33.33±2.89	21.67±2.89	41.33±2.89
10^3	13.33±2.89	28.33±7.67	20.00±0.00	43.33±7.64	23.33±2.89	48.33±7.64
10^4	13.33±5.77	36.67±2.89	18.33±5.77	45.00±5.00	23.33±2.89	56.67±7.64
10^5	15.00±5.00	41.67±2.89	18.33±5.77	60.00±5.00	21.67±7.64	78.33±5.77
10^6	15.00±8.66	51.67±5.77	20.00±8.66	85.00±8.66	25.00±10.00	100.00±0.00

SD = Standard Deviation

Table 5: Cumulative mortality (%) in *P. pelagicus* larvae (Zoea1 Z1) after exposure to different doses (cfu mL^{-1}) of *P. piscicida* isolate from the gut (mean±SD)

	Mortality (%)					
Dose	Control	24 h	Control	48 h	Control	72 h
10^2	13.33±2.89	26.67±2.89	18.33±2.89	40.00±5.00	21.67±02.89	51.67±5.77
10^3	11.67±2.89	35.00±10.0	16.67±2.89	53.33±2.89	25.00±08.66	68.33±7.64
10^4	11.67±2.89	51.67±5.77	18.33±5.77	73.33±2.89	23.33±10.41	93.33±7.64
10^5	13.33±5.77	73.33±2.89	18.33±5.77	98.33±2.89	23.33±07.64	100.00±0.00
10^6	11.67±7.64	100.00±0.00	16.67±2.89	100.00±0.00	21.67±02.89	100.00±0.00

SD = Standard Deviation

Table 6: Cumulative mortality (%) in *P. pelagicus* larvae (Zoea1 Z1) after exposure to different doses (cfu mL^{-1}) of *S. epidermidis* isolate from the gut (mean±SD)

	Mortality (%)					
Dose	Control	24 h	Control	48 h	Control	72 h
10^2	13.33±2.89	21.67±2.89	20.00±5.00	28.33±2.89	23.33±2.89	36.67±7.77
10^3	13.33±7.64	23.33±2.89	18.33±5.77	30.00±5.00	25.00±5.00	36.67±5.77
10^4	11.67±2.89	30.00±5.00	16.67±5.77	36.67±2.89	21.67±5.77	43.33±7.64
10^5	11.67±7.64	35.00±5.00	18.33±5.77	41.33±2.89	20.00±5.00	45.00±5.00
10^6	13.33±5.77	38.33±2.89	21.67±2.89	45.00±0.00	26.67±7.64	51.67±5.77

SD = Standard Deviation

Table 7: Cumulative mortality (%) in *P. pelagicus* larvae (Zoea1 Z1) after exposure to different doses (cfu mL^{-1}) of *M. luteus* isolate from the gut (mean±SD)

	Mortality (%)					
Dose	Control	24 h	Control	48 h	Control	72 h
10^2	13.33±2.89	18.33±2.89	20.00±5.00	23.33±2.89	25.00±8.66	31.67±2.89
10^3	15.00±5.00	21.33±2.89	18.33±2.89	28.33±5.77	25.00±5.00	31.67±2.89
10^4	11.67±7.64	21.33±2.89	18.33±5.77	35.00±8.66	23.33±2.89	40.00±8.66
10^5	15.00±0.00	30.00±5.00	20.00±5.00	36.67±2.89	23.33±2.89	41.67±2.89
10^6	13.33±5.00	35.00±8.66	21.67±2.89	40.00±5.00	25.00±5.00	48.33±2.89

SD = Standard Deviation

dose 10^6 cfu mL^{-1} produced 51.67, 85.0 and 100% mortality after 24, 48 and 72 h post dose, respectively (Table 4). *P. piscicida* at dose 10^2, 10^3, 10^4 and 10^5 cfu mL^{-1} produced mortality 51.67, 68.33, 93.33 and 100%, respectively after 72 h post dose and 10^6 cfu mL^{-1} caused 100% mortality within 24 h challenge dose. Pathogenic effects of *P. piscicida* after 72 h were observed more severe at 10^3 cfu mL^{-1} in comparisons to *V. harveyi* and *V. parahaemolyticus;* it produced 68.33% mortality while *V. harveyi* and *V. parahaemolyticus* produced 61.67 and 48.33%, respectively. Similar to *V. harveyi*, the doses 10^6 and 10^5 cfu mL^{-1} indicated 100% mortality after 24 and 72 h, respectively (Table 5). Overall results of pathogenicity showed that *P. piscicida* and *V. harveyi* were more pathogenic microbes in comparisons to other isolates of the gut of *P. pelagicus*. However, *S. epidermidis* at inoculation doses 10^2, 10^3, 10^4 and 10^5 cfu mL^{-1} produced mortality 36.67, 36.67, 43.33, 45.00 and 51.67%, respectively (Table 6). Moreover, *M. luteus* at inoculation 10^2, 10^3, 10^4 and 10^5 cfu mL^{-1} produced mortality 31.67, 31.67, 40.00, 41.67 and 48.33%, respectively (Table 7). In the present study, *S. epidermidis* and *M. luteus* isolates were observed as weak pathogens. During the experimental challenges, escalated mortalities were observed at each increased dose. Larvae of *P. pelagicus* showed symptoms such as weariness, loss of equilibrium, gyratory movement and general weakness within 3-6 h after challenge with inoculated bacteria. Comparative mortality of all pathogenic isolates has been shown in Fig. 1. A natural mortality based on 24-72 h experiments was observed ranges between 11.67-26.67% in all controls employed.

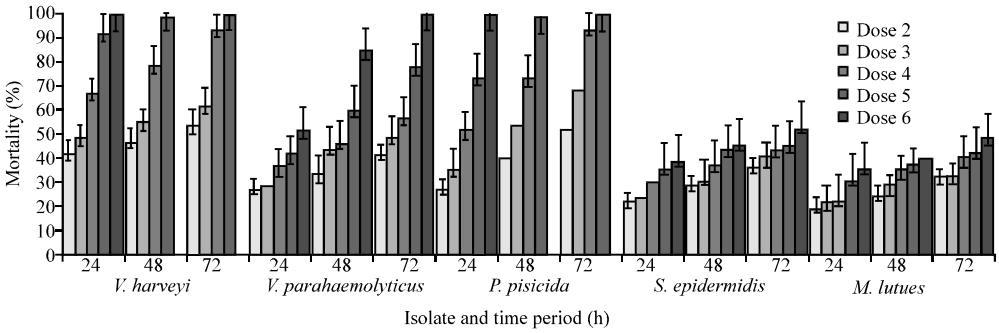

Fig. 1: Comparative cumulative mortality (%) of *Portunus pelagicus* larvae exposed to different doses of isolates from the gut (Dose: 2 = 10^2, 3 = 10^3, 4 = 10^4, 5 = 10^5 and 6 = 10^6)

Table 8: Antibiotic sensitivity profile of the gut isolates of *P. pelagicus*

	V. harveyi (n = 180)			*V. parahaemolyticus* (n = 180)			*P. piscicida* (n = 119)			*S. epidermidis* (n = 88)			*M. luteus* (n = 95)		
Antibiotics	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Co-trimoxazole	107	13	60	146	8	26	2	11	106	0	0	88	0	0	95
Erythromycin	149	18	13	16	12	152	11	5	103	0	0	88	0	0	95
Streptomycin	161	9	10	122	11	47	5	7	107	0	0	88	0	0	95
Oxytetracycline	76	18	86	46	32	102	22	3	94	0	0	88	0	0	95
Ampicillin	67	0	113	57	14	109	2	21	96	0	0	88	0	0	95
Nystatin (Mycostatin)	160	20	0	157	11	12	79	22	18	0	0	88	0	0	95
Chloramphenicol	46	7	127	12	0	168	0	3	116	0	0	88	0	0	95
Gentamicin	57	16	107	35	0	145	0	2	117	0	0	88	0	0	95
Penicillin	79	3	98	53	31	96	96	23	0	0	0	88	0	0	95
Kanamycin	96	2	82	167	4	9	0	17	102	0	0	88	0	0	95
Furazolidone	146	0	34	152	0	28	56	0	63	0	0	88	0	0	95
Ciprofloxacin	76	0	104	34	0	146	29	0	90	0	0	88	0	0	95
Rifampicin	156	0	24	88	0	92	45	0	74	0	0	88	0	0	95
Neomycin	149	0	31	144	0	36	39	0	80	0	0	88	0	0	95

S = Sensitive; I = Intermediate; R = Resistant

This pathogenic study was based on pathogenic microbes isolated from one female specimen. The mean differences among various pathogenic doses were statistically significant ($p<0.05$). The LD_{50} of *V. harveyi* was 1.2×10^3 cfu mL^{-1} (24 h), *V. parahaemolyticus* was 9.6×10^5 cfu mL^{-1} (72 h), *P. piscicida* was 9.8×10^3 cfu mL^{-1} (24 h) and *S. epidermidis* was 9.8×10^5 cfu mL^{-1} (72 h), moreover *M. luteus* did not produce mortality above the 50% during challenge doses therefore, no LD_{50} was calculated. *V. parahaemolyticus* and *S. epidermidis* indicated low virulence during the challenge doses.

In present study, total 662 isolates pathogens including 180 *V. harveyi* and *V. parahaemolyticus* isolates, respectively and *P. piscicida* 119, S. *epidermidis* 88 and *M. luteus* 95 were tested against 14 various antibiotics for susceptibility. We observed that *V. harveyi*, *V. parahaemolyticus* and *P. piscicia* isolates showed antimicrobial resistance and sensitivity against more than one antimicrobial agent tested (Table 8 and 9). *V. harveyi* showed multiple resistant to all 14 antibiotics tested and it showed susceptible response to 13 antibiotics and did not show any sensitivity to Nystatin. The pervasiveness of resistance of *V. harveyi* isolates was higher to streptomycin (89.44%), nystatin (88.89%), rifampicin (86.67%), erythromycin (82.78%), neomycin (82.78%), furazolidone (81.11%) followed by co-trimoxazole (59.45%), kanamycin (53.33%), penicillin (43.89%), oxytetracycline (42.22%), ciprofloxacin (42.22%), ampicillin (37.22%), gentamicin (31.67%) and chloramphenicol (25.55%). However, *V. harveyi* showed higher susceptibility to chloramphenicol (70.55%), ampicillin (62.78%) followed by gentamicin (59.44%), ciprofloxacin (57.78%), penicillin (54.44%), oxytetracycline (47.78%), kanamycin (45.55%), co-trimoxazole (33.33%), furazolidone (18.89%) and neomycin (17.22%). This organism was least susceptible rifampicin (13.33%), erythromycin (7.22%) and streptomycin (5.56%). *V. harveyi* showed an intermediate response to nine antibiotics tested highest to nystatin 11.11% and least to kanamycin (1.11%) (Table 8 and 9). *V. parahaemolyticus* was highly resistant to kanamycin (92.78%), nystatin (87.22%), furazolidone (84.44%), co-trimoxazole (81.11%), neomycin (80%), respectively followed by streptomycin (67.78%), rifampicin (48.89%) ampicillin (31.67%), penicillin (29.44%), oxytetracycline (25.55%), gentamicin

Table 9: Percentage wise profile of antibiotic sensitivity of the gut isolates of *P. pelagicus*

	V. harveyi (n = 180)			*V. parahaemolyticus* (n = 180)			*P. piscicida* (n = 119)			*S. epidermidis* (n = 88)			*M. luteus* (n = 95)		
Antibiotics	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Co-trimoxazole	59.45	7.22	33.33	81.11	4.44	14.45	1.68	9.24	89.08	0	0	100	0	0	100
Erythromycin	82.78	10.00	7.22	8.89	6.67	84.44	9.25	4.20	86.55	0	0	100	0	0	100
Streptomycin	89.44	5.00	5.56	67.78	6.11	26.11	4.20	5.88	89.92	0	0	100	0	0	100
Oxytetracycline	42.22	10.00	47.78	25.55	17.78	56.67	18.49	0.00	81.51	0	0	100	0	0	100
Ampicillin	37.22	0.00	62.78	31.67	7.78	60.55	1.68	17.65	80.67	0	0	100	0	0	100
Nystatin	88.89	11.11	0.00	87.22	6.11	6.67	66.39	18.49	15.12	0	0	100	0	0	100
Chloramphenicol	25.55	3.89	70.55	6.67	0.00	93.33	0.00	2.52	97.48	0	0	100	0	0	100
Gentamicin	31.67	8.89	59.44	19.44	0.00	80.56	0.00	1.68	98.32	0	0	100	0	0	100
Penicillin	43.89	1.67	54.44	29.44	17.22	53.34	80.67	19.33	0.00	0	0	100	0	0	100
Kanamycin	53.33	1.11	45.55	92.78	2.22	5.00	0.00	14.29	85.71	0	0	100	0	0	100
Furazolidone	81.11	0.00	18.89	84.44	0.00	15.56	47.06	0.00	52.94	0	0	100	0	0	100
Ciprofloxacin	42.22	0.00	57.78	18.89	0.00	81.11	24.37	0.00	75.63	0	0	100	0	0	100
Rifampicin	86.67	0.00	13.33	48.89	0.00	51.11	37.82	0.00	62.18	0	0	100	0	0	100
Neomycin	82.78	0.00	17.22	80.00	0.00	20.00	32.77	0.00	67.23	0	0	100	0	0	100

S = Sensitive; I = Intermediate; R = Resistant

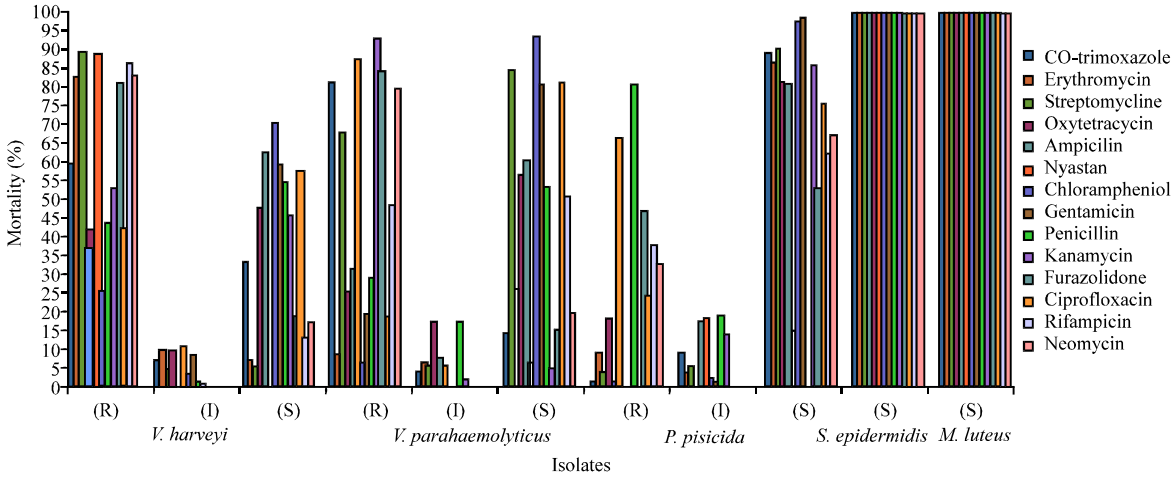

Fig. 2: Antibiogram of the gut isolates

(19.44%), ciprofloxacin (18.89%) and it was least resistant to erythromycin (8.89%), chloramphenicol (6.67%). Whereas, this organism was highly susceptible to chloramphenicol (93.33%), erythromycin (84.44%), ciprofloxacin (81.11%) gentamicin (80.56%) followed by ampicillin (60.55%), oxytetracycline (56.67%), penicillin (53.33%), rifampicin (51.11%), streptomycin (26.11%) and showed least resistance to neomycin (20%), furazolidone (15.56%), co-trimoxazole (14.45%), nystatin (6.67%) and kanamycin (5%), respectively. *V. parahaemolyticus* showed intermediate response to eight antibiotics higher to oxytetracycline (17.78%) and penicillin (17.22%) respectively and least to kanamycin (2.22%) (Table 8 and 9). *P. piscicida* was highly resistance to penicillin (80.67%) (intermediate response (19.33%) but not susceptible), nystatin (66.39%) followed by furazolidone (47.06%), rifampicin (37.82%), neomycin (32.77%), ciprofloxacin (24.37%) and oxytetracycline (18.49%), respectively and it showed slightest resistant to erythromycin (9.25%), streptomycin (4.20%), co-trimoxazole (1.68%) and ampicillin (1.68) and none of *P. piscicida* showed resistant chloramphenicol, gentamicin and kanamycin.

In major extent *P. piscicida* was susceptible to gentamicin (98.32%), chloramphenicol (97.48%), streptomycin (89.92%), co-trimoxazole (89.08%), erythromycin (86.55%), kanamycin (85.71%), oxytetracycline (81.51%), ampicillin (80.67%), ciprofloxacin (75.63%), neomycin (67.23%), rifampicin (62.18%) and furazolidone (52.94%) and least susceptible to nystatin (15.12%).

This organism was not determined as sensitive to penicillin. But it showed intermediate response to 9 antibiotics highest to penicillin (19.33%) and least to erythromycin (4.2%) (Table 8 and 9). *S. epidermidis* and *M. luteus* were susceptible to all 14 antibiotics tested (Table 8 and 9). Antibiogram of all pathogenic isolates is show in Fig. 2.

DISCUSSION

The female crabs collected from Strait of Tebrau Johor, Malaysia which are usually used as broodstock for larvae rearing as well as for food consumption locally. The present bacteriology study revealed that the guts of 80 female were harbouring fish/shellfish pathogenic diversity of bacteria including *V. harveyi*, *V. parahaemolyticus*, *P. piscicida*, *M. luteus* and *S. epidermidis*. Pathogenicity all five microbes of the gut were undertaken with various inoculation doses against Zoea 1 (Z1 larvae stage) of *P. pelagicus*.

Pathogenic *V. harveyi* found globally in marine environments is a serious pathogen for a wide range of marine animals. With the rapid developments in aquaculture particularly in Asia and South America, the organism has become recognized as a serious cause of disease particularly of marine invertebrates (Austin and Zhang, 2006). The Vibriosis bacteria have caused high mortality in cultured shrimp worldwide (Lightner and Lewis, 1975; Lightner *et al*., 1992; Lavilla-Pitogo *et al*., 1996). *V. harveyi* was more pathogenic in comparison to the other luminous bacteria causing mass deaths in crab larvae (Parenrengi *et al*., 1993). In other study Won and Park (2008) reported that *V. harveyi* strains were considerably more pathogenic to black rockfish than to olive flounder in both live bacteria and ECPs experimental challenges. Experimental challenges in the present study, *V. harveyi* was observed as pathogenic even at low dose of inoculation 10^2 cfu mL^{-1} produced 53.33% mortality after 72 h post dose. However, higher doses of inoculation 10^3, 10^4 and 10^5 cfu mL^{-1} produce 61.67, 93.33 and 100% mortality after 72 h post dose, respectively while dose 10^6 cfu mL^{-1} was more severe which killed all larvae in 24 h post dose (Table 3). Selvin *et al*. (2005) observed 100% deaths within 6 h post dose to *P. monodon* juvenile at inoculation 10^8 cfu $shrimp^{-1}$ of *V. harveyi*. Abraham (2006) also observed 100% mortality with mysis 3 larvae of Indian white shrimp, *Fenneropenaeus indicus* after 72 h post dose at 10^6 cfu mL^{-1} of *V. harveyi*. Karunasagar *et al*. (1994) challenged *V. harveyi* against postlarvae of *P. monodon*, he observed 100% mortality at 10^5 cfu mL^{-1} day 5 post dose. In other study Lavilla-Pitogo *et al*. (1990) reported that *V. harveyi* has been widely recognized as a primary pathogen of many commercially cultured invertebrate species the world over and the mortality of larval stages protozoea to postlarvae of *P. monodon* shrimps due to *V. harveyi* in the hatcheries often reaches 100%. Talpur *et al*. (2011) observed 100% mortality of Z1stage larvae of *P. pelagicus* at inoculation 10^6 cfu mL^{-1} after 24 h post dose of different *V. harveyi* isolates isolated from larval rearing system. Results of present study are in agreement with previous reports by various researchers elsewhere that *V. harveyi* is a well recognised potential pathogen which cause major infections in the larvae and resulted in mass mortality. Pathogenic challenges proved the pathogenicity of *V. harveyi* even inculcation at low dose produced stern mortality. Moreover pathogenicity of *V. harveyi* has shown serious concern in larval experimental dose and cumulative mortality was observed with the increase dose potency.

It is now widely considered that the *V. parahaemolyticus* is an emerging fish pathogen and has been associated with mortalities in Iberian toothcarp, *Aphanius iberus* with the signs centring on external haemorrhages and tail rot (Austin and Austin, 2007) and it was recovered from diseased milkfish, *Chanos chanos* in the Philippines (Austin and Austin, 2007). Results of present study show that the pathogenicity of *V. parahaemolyticus* was bit less pathogenic in comparison to *V. harveyi* and *P. piscicida*. Pathogenic dose at inoculation 10^2 cfu mL^{-1} produced 41.33% mortality while inoculation doses 10^3, 10^4, 10^5 and 10^6 cfu mL^{-1} produced 48.33, 56.67, 78.33 and 100%, respectively after 72 h post challenge dose (Table 4). Sudhesh and Xu (2001) observed, 100% mortality after 7 days when injected with a *V. parahaemolyticus* dose of 1×10^8 cfu *Penaeus monodon* shrimp whereas mortalities of 80, 20 and 10% were obtained with 10^6, 10^4 and 10^2, respectively. Aguirre-Guzman *et al*. (2001) infected American White Shrimp *Litopenaeus vannamei* larval sub stages from nauplii to mysis 3 separately by immersion for 30 min in 100 mL sterilized seawater with the corresponding bacterial suspensions (10^3, 10^5 or 10^7 cfu mL^{-1}) of *V. parahaemolyticus* and *V. harveyi*. He observed that *V. harveyi* and *V. parahaemolyticus* showed high mortality rates for all shrimp larval substages at 10^5 and 10^7 cfu mL^{-1} with the highest mortality rate at 10^7 cfu mL^{-1}. In other development by Alapide-Tendencia and Dureza (1997) suggested that *V. harveyi* and *V. parahaemolyticus* were responsible for the red disease syndrome in *P. monodon* juveniles when they were presented at dose levels of 10^5-10^7 cfu mL^{-1}. Robertson *et al*. (1998) reported that infection of *P. vannamei* larvae with *V. harveyi* at 10^5 cfu mL^{-1} produced a larval disease called bolitas negricans (a local name from Ecuador) and bioluminescence. Literature depicts that *V. parahaemolyticus* is a well-recognized pathogen of invertebrates including larvae of abalone, *Haliotis diversicolor supertexta* (Cai *et al*., 2007) and in *P. monodon*, the organism has been implicated as a cause of red disease in India shrimp (Jayasree *et al*., 2006). In present study, we also observed low mortality with lower dose and elevated dose produced high mortality. Moreover, *V. harveyi* and *V. parahaemolyticus* were observed effective pathogen in experimental challenges.

The pathogenic nature of *P. piscicida* bacterium is scantily documented in literature. Nelson and Ghiorse (1999) reported that an isolate *P. piscicida* Cura-d was

associated with the highest mortality of both *Amphiprion clarkii* (Bennett) and *Amphiprion curacao* (Bloch) eggs. The majority of the eggs die within 24-36 h. In the present study, it was observed the *P. piscicida* was more pathogenic in comparison to *V. parahaemolyticus*. Experimental challenge produced 100% deaths at inoculation 10^6 and 10^5 cfu mL^{-1} after 24 and 72 h post dose, respectively (Table 5). Hansen *et al.* (1965) reported that *P. piscicida* appeared to be toxic to certain species of fish including *Lutjanus apodus* (schoolmaster), *Eucinostomus pseudogula* (sand perch), *Fundulus similis* (killifish) and *Mollienesia latipinna* (mollie) as well as the fiddler crabs, *Uca pugnas* and *Uca pugilator*. A toxic syndrome appears within a few hours and death follows quickly after neuromuscular effects appear. The study confirmed that *P. piscicida* has a pathogenic and virulent effect on larval survival resulting in severe mortalities in inoculation challenge doses. This study substantiated the observation given by Hansen *et al.* (1965) regarding the toxicity of *P. piscicida*. Prevalence of this organism has serious concern in hatchery system for seed production of *P. pelagicus* crab. In the present study, it was observed that pathogenic effects of 10^5 cfu mL^{-1} were higher after 72 h post inoculation in contrast to *V. harveyi* at inoculation 10^3 cfu mL^{-1} *P. piscicida* produced 68.33% while *V. harveyi* produced 61.67% mortality.

In literature cited *S. epidermidis* was 1st reported as a fish pathogen by Kusuda and Sugiyama (1981) in farmed yellow tail, *Seriola quinquiradiata* and red sea bream, *Chrysophrus major* in Japan. *S. epidermidis* caused mass mortality of cultured Tilapia in Taiwan (Huang *et al.*, 1999). *S. epidermidis* caused infection in gillhead sea bream, *Sparus aurata* juvenile (3-5 g) in a net cage and outbreak resulted fish loses upto 12% in 1 day (Kubilay and Ulukoy, 2004). In the present study, *S. epidermidis* showed some pathogenic properties in experimental challenges at 10^6 cfu mL^{-1} it produced 51.67% mortality after 72 h post dose.

M. luteus has been reported to be another pathogen in aquaculture life. *M. luteus* caused Rainbow Trout Fry Syndrome (RTFS) (Austin and Stobie, 1992). *M. luteus* showed weak response and resulted in 48.33% mortality after 72 h exposure challenge. Both *S. epidermidis* and *M. luteus* were responded as weak pathogens in comparisons to *V. harveyi*, *V. parahaemolyticus* and *P. piscicida*.

Selection of Zoea-1 (Z1) for pathogenic challenges was in the context because it is the 1st stage larva which is more faint and susceptible to pathogenic hazards. The aim of study was to observe the larvae at this stage are infected could they survive or not. It was noticed that even low dose inoculation 10^2 cfu mL^{-1} of pathogens, infected the larvae and with the time passage their deaths were mounted. Lavilla-Pitogo *et al.* (1990) reported that challenge doses at concentration 10^{-2} cfu mL^{-1} of *V. harveyi* have caused 100% mortality in *P. monodon* larvae. We also believe that whenever larvae are infected by causative pathogen at an early stage; it is hard them to survive further. Within the provided environment, the pathogens are multiplied frequently because of available nutrients and their strength is rising up which definitely perilous to larvae life. One common factor was observed in the present study that with the increase in dose concentration the mortality was cumulative at each dose and was mounted with time interval. Larval substage Z1 of *P. pelagicus* was highly susceptible to infections of *V. harveyi* and *P. piscicida* at corresponding dose 10^6 and 10^5 cfu mL^{-1} showed 100% mortality during challenge doses whereas *V. parahaemolyticus* at corresponding dose 10^6 cfu mL^{-1} exposed 100% mortality after 72 h post dose.

The outbreaks caused by *V. harveyi* have been reported in many marine fishes (Soffientino *et al.*, 1999; Zhang and Austin, 2000) and over a wide geographical range (Sunaryanto and Mariam, 1986; Lavilla-Pitogo *et al.*, 1990; Won *et al.*, 2006). We also confirmed the pathogenicity of the isolates to *P. pelagicus* larvae using a challenge test. The *V. harveyi* and *P. piscicida* isolates were considerably more pathogenic to larvae of *P. pelagicus*. Particularly, both of the tested isolates were strong pathogenic with LD_{50} values of 1.2×10^3 cfu mL^{-1} (24 h) and 9.8×10^3 cfu mL^{-1} (24 h), respectively. Karunasagar *et al.* (1994) reported that the LD_{50} estimate of pathogenic *V. harveyi* was $2\text{-}5\times10^3\text{-}1.5\times10^5$ cfu mL^{-1} for *P. monodon* larvae but we found the LD_{50} of *V. harveyi* was 1.2×10^3 cfu mL^{-1} (24 h). Similarly, Thakur *et al.* (2003) observed LD_{50} estimate of pathogenic *V. parahaemolyticus* was 5.99×10^5 cfu mL^{-1} *P. monodon* and reported as low virulent pathogen and we observed the same that the LD_{50} for *V. parahaemolyticus* was 9.6×10^5 cfu mL^{-1} (72 h) indicating low virulent as compared to *V. harveyi* and *P. piscicida* isolates. The pathogenic property of *Pseudoalteromonads* to aquatic organisms is little known. *P. piscicida* Cura-d was pathogenic to eggs of damselfish (Pomacentridae) species, *Amphiprion clarkia* (Nelson and Ghiorse, 1999) but no LD_{50} results are shown in literature regarding *P. piscicida*. We observed that LD_{50} estimate of *P. piscicida* was 9.8×10^3 cfu mL^{-1} (24 h) exposed to *P. pelagicus* larvae while *S. epidermidis* was indicating response as weak pathogen and LD_{50} was determined 9.8×10^5 cfu mL^{-1} (72 h).

The pathogenic experimental challenges of *V. harveyi*, *V. parahaemolyticus*, *P. piscicida*, *M. luteus* and *S. epidermidis* to the larvae of *P. pelagicus* is the first ever study which indicate to the probable role of virulence determinants of the bacteria isolated from the gut of female crab.

The susceptibility tests of 662 isolates from 180 female guts including *V. harveyi* (n = 180), *V. parahaemolyticus* (n = 180), *P. piscicida* (n = 119), *S. epidermidis* (n = 88) and *M. luteus* (n = 95) were tested for antimicrobial tests against 14 antibiotics during the present study. Researchers found that *V. harveyi* *V. parahaemolyticus* and *P. piscicida* isolates showed multiple antimicrobial resistances against antimicrobial agents tested. However, *S. epidermidis* and *M. luteus* were susceptible to all 14 antibiotics tested.

The marine-estuarine bacterium *V. harveyi* is an important pathogen of invertebrates which results in severe mortality. Vibriosis is one of the most frequent diseases affecting crustaceans, fishes and molluscs. To treat such infections, it is a common practice to employ antibiotics such as oxytetracycline, cholramphenicol and other drugs. In the present study, data on antibiotic resistance indicates that all the isolates of the gut of female crab *P. pelagicus* were resistant to majority of examined antibiotics.

Resistance of marine fish and shrimp pathogenic bacteria to commonly used antibiotics has been reported before throughout the world (Chowdhury *et al*., 1989; Schmidt *et al*., 2000). Ansari and Raissy (2010) found the incidence to *V. harveyi* and *V. parahaemolyticus* isolated from Lobster, *Panulirus homarus* showed resistance towards ampicillin, penicillin, streptomycin, erythromycin and tetracycline. Another researcher Parvathi *et al*. (2011), reported *V. harveyi* isolated from shrimp hatchery and many of isolates were 100% resistant to various drugs such as erythromycin, kanamycin, penicillin and streptomycin (92%).

V. harveyi isolate from *P. monodon* was resistant to variety of antibiotics including ampicillin, cholramphenicol, erythromycin, penicillin-G, co-trimoxazole and streptomycin (Aftabuddin and Akter, 2010). *V. harveyi* isolated from diseased *P. monodon* showed antibiotic resistivity pattern to co-trimoxazole, nystatin, penicillin-G and it was intermediate to variety of drugs Selvin *et al*. (2005). Srinivasan and Ramasamy (2009) observed antibiotic resistance patterns of *V. harveyi* associated with diseased shrimp of aquaculture environment and they reported that *V. harveyi* was 100% resistant to ampicillin, erythromycin, penicillin g and furazolidone and streptomycin and rifampicin, 72.73% to oxytetracycline, 27.27% to neomycin, cholramphenicol and gentamicin, 18.18% to ciprofloxacin, respectively. Adeleye *et al*. (2008) reported *Vibrio* species isolated from seafoods among them *V. harveyi* and *V. parahaemolyticus* were 100% resistant to amoxicillin, cholramphenicol , gentamicin, tetracycline and 64.3% *V. harveyi* were resistant to co-trimoxazole.

We observed that the antibiogram of *V. harveyi* showed mixed patterns of resistance to all 14 antibiotics used for susceptibility study. *V. harveyi* was resistant to all tested antibiotics such as streptomycin (89.44%), nystatin (88.89%), rifampicin (86.67%), erythromycin (82.78%), neomycin (82.87%), furazolidone (81.11%), co-trimoxazole (59.45%) kanamycin (53.33%), penicillin (43.89%), oxytetracycline (42.22%), ciprofloxacin (42.22%), ampicillin (37.22%), gentamicin 31.67%) and chloramphenicol (25.55%) (Table 9). The results of antibiotic tests fairly matched with the study of Aftabuddin and Akter (2010), Selvin *et al*. (2005), Adeleye *et al*. (2008), Srinivasan and Ramasamy (2009), Ansari and Raissy (2010) and Parvathi *et al*. (2011). But to some extent the results quite differ from the findings of Aftabuddin and Akter (2010), Selvin *et al*. (2005), Parvathi *et al*. (2011), Srinivasan and Ramasamy (2009), Adeleye *et al*. (2008) because we did not find any *V. harveyi* 100% resistant to any drug tested as previously reported by these researchers. The variations in the results owing to investigations of microbes isolated from different samples of the gut specimens of female crab, *P. pelagicus* round the year. We observed during the present study that *V. harveyi* was highly resistant to streptomycin (89.44%) followed by nystatin (88.89%) and was susceptible to 13 antibiotics in major or least pattern but it was not susceptible to only one drug nystatin (Table 9). Out of 13 antibiotics *V. harveyi* showed high susceptible response to chloramphenicol (70.55%) drug only. Sengupta *et al*. (2003) reported that *V. harveyi* (n = 60) isolated from shrimps farms of west bengal, india were found resistant to nystatin (100%), co-trimoxazole (96%), gentamicin (82.28%), ciprofloxacin (59.43%) and oxytetracycline (40.57%). The findings are closely in agreement with the results of Sengupta *et al*. (2003).

We found in present study that *V. parahaemolyticus* was resistant all 14 drugs tested with higher to 6 and moderate or least to 8 drugs (Table 9). Previous studies have shown that streptomycin, rifampicin, kanamycin, tetracycline, polymyxin B were active against *Vibrio* sp. (Li *et al*., 1999; Ottaviani *et al*., 2001). However, Ottaviani *et al*. (2001) observed that *V. parahaemolyticus* was resistant to penicillin, ampicillin, kanamycin and rifampicin. Besides, their results also showed that increase of salt concentration cause the change of sensitivity toward antibiotics from the resistant to susceptible phenotype. Thakur *et al*. (2003) found that *V. parahaemolyticus* was resistant to oxytetracycline. Oxytetracycline is most common antibiotic widely used in aquaculture. This antibiotic seems to be successful in controlling vibriosis in shrimp culture, even though laboratory results showed that the bacteria are resistant to oxytetracycline (Tendencia and de la Pena, 2001. Zulkifli *et al*. (2009) reported the out of 32 strains isolated from cockles and tested; they found >50% of strains were resistant to penicillin, ampicillin and streptomycin drugs.

The occurrence of ampicillin resistance Vibrio isolates in marine environments are generally high (Ferrini *et al*., 2008; Han *et al*., 2007). In other study, Srinivasan and Ramasamy (2009) reported that *V. parahaemolyticus* associated with aquaculture environment was resistant to antibiotic drugs ampicillin, erythromycin, penicillin G, furazolidone, streptomycin, rifampicin and oxytetracycline. Most recently Oh *et al*. (2011) reported that *V. parahaemolyticus* isolated from the farmed fish in Korea was resistant to various drugs such as ampicillin (52.2%), streptomycin (7.2%), gentamicin (1.8%), nystatin (1.4%), chloramphenicol (3.7%), tetracycline (3.7%), rifampicin (11.9%) and erythromycin (0.9%). His study was based on 218 isolates. Han *et al*. (2007) observed high percentage of *V. parahaemolyticus* with reduced susceptibility to ampicillin suggests a potential for low efficiency of ampicillin in empirical treatment of *V. parahaemolyticus* infections. In the present study, we observed that *V. parahaemolyticus* responded higher resistance to kanamycin (92.78%), nystatin (87.22%), furazolidone (84.44%), co-trimoxazole (81.11%), neomycin (80%), streptomycin (67.78%) followed by rifampicin (48.89%), ampicillin (31.67%), penicillin (29.44%) and oxytetracycline (25.50%) and it was not observed 100% sensitive to any of the drug tested. Present study evident that *V. parahaemolyticus* was highly susceptible to chloramphenicol (93.33), erythromycin (84.44%), ciprofloxacin (81.11%), gentamicin (80.56%), ampicillin (60.55%), oxytetracycline (56.67%), penicillin (53.33%) and rifampicin (51.11%) while it was least susceptible to co-trimoxazole, nystatin, streptomycin, furazolidone, neomycin and kamamycin. Out of 14 drugs tested so far *V. parahaemolyticus* did not show any intermediated response to chloramphenicol, gentamicin, furazolidone, ciprofloxacin, rifampicin and neomycin while it responded least intermediate pattern to rest of drugs (Table 9). The results are closely in accord to findings of Ottaviani *et al*. (2001), Thakur *et al*. (2003), Adeleye *et al*. (2008), Zulkifli *et al*. (2009), Ferrini *et al*. (2008), Han *et al*. (2007), Srinivasan and Ramasamy (2009) and Oh *et al*. (2011). But we did not observe any of *V. parahaemolyticus* isolate either 100% resistant or susceptible to any of the drug tested during the present study. The variation in results was owing to variety of isolates (662) from 180 gut specimens were tested and would have different susceptible acceptance.

Hansen *et al*. (1965) observed *P. piscicida* was not sensitive to tetracycline and penicillin and sensitive to chloramphenicol, erythromycin, novobiocin and kanamycin. We found that *P. piscicida* was highly sensitive to three antibiotics tested including gentamicin (98.32%), chloramphenicol (93.33%) and kanamycin (85.71 and 14.29% intermediate but not sensitive) and it showed resistance to all antibiotics tested, highly to penicillin (80.67%) and nystatin (66.39%) and moderate to furazolidone (47.06), rifampicin (37.82%) and neomycin (32.77%) and was least resistance to rest of antibiotics (Table 9). None of *P. piscicida* was observed susceptible to penicillin and least to nystatin (15.12%) and was highly susceptible to rest of drugs (Table 9). The results more or less are matching to the findings of Hansen *et al*. (1965) but we used more drugs during the present study and tested diversity of isolates isolated round the year from the specimens of female guts.

However, *S. epidermidis* isolates and *M. luteus* isolates were susceptible to all 14 antibiotics tested. Result of this study was similar to Huang *et al*. (1999) where they found four strains of *S. epidermis* were susceptible to all drugs and showed resistance to Sulfadiazine only among the antibiotic tested. We did not tested Sulfadiazine drug in the present study.

It was observed that *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* showed multi resistant and multi susceptibility to various kinds of antibiotics tested. The most frequently observed pattern of multi resistance among *V. harveyi and V. parahaemolyticus* and *P. piscicida* was vary from drug to drug. From the results of 14 antibiotics tested, we observed that the highest frequency of single drug resistance in *V. harveyi* was streptomycin (89.44%) and sensitive to chloramphenicol (70.55%). Similarly, the highest frequency of single-drug resistance in *V. parahaemolyticus* was to kanamycin (92.78%) and sensitive to chloramphenicol (93.33%). The highest frequency of single drug resistance in *P. piscicida* was to Penicillin (80.67+19.33% intermediate but no sensitive) and sensitive to gentamicin (98.32%).

To date knowledge, there is no report available on the pathogenicity and multiple antibiotic resistances of bacterial pathogens isolated from the gut of *P. pelagicus* of Johor, Malaysia. However, the results of the present study serve as a baseline data for future research on the extent of pathogenicity and antibiotic resistance in larviculture of *P. pelagicus* and improve the knowledge on drug resistant strains and their effect on future therapy of *P. pelagicus* as well as human diseases.

CONCLUSION

Results of the present study further support the view that antibiotic-resistant *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* isolated from the gut of female crab caused the high mortality in *P. pelagicus* larvae during experimental challenges. Infections caused by the gut pathogens have serious consequences in larviculture of *P. pelagicus* and therapeutic use of tested antibiotic is

questionable. The data show that early larval stage (Z 1) had sensitivity to dosages of bacterial isolates and mortality showed directly proportionate with increase in dose concentrations. The virulence factors of isolated pathogenic species affecting *P. pelagicus* larvae are not known in detail. It is appropriate that further research is justified to clarify the nature of the pathogenicity mechanism of these microbes in detail.

RECOMMENDATIONS

Therefore, deceitful use of antibiotics against diseases should be avoided and restrictions for the use of antibiotics may be implemented by a nationwide antibiotic policy for Malaysia. Further it is suggested from the results that the use of antibiotics should be strictly controlled either used in hatcheries or farms to prevent the dissemination of antibiotic-resistant bacteria.

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